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Silicon carbide fiber-mediated DNA delivery into plant cells

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Summary. Silicon carbide fiber-mediated delivery of DNA into intact plant cells was investigated. Black Mexican Sweet (BMS) maize (*Zea mays*) and tobacco (*Nicotiana tabacum*) suspension culture cells were vortexed in the presence of liquid medium, plasmid DNA encoding β-glucuronidase (GUS), and silicon carbide fibers. Penetration of BMS cells by the silicon carbide fibers was observed by scanning electron microscopy of vortexed cells. Following fiber and DNA treatment, BMS cells transiently expressed GUS activity at a mean frequency of 139.5 units (one unit = one blue cell or one colony of blue cells) per sample. Treated tobacco cells expressed an average of 373 GUS units per sample. Untreated controls did not exhibit GUS activity. These results indicate that the silicon carbide fibers-vortex procedure can be used to rapidly and inexpensively deliver foreign DNA into intact plant cells for investigations of transient gene expression.

Abbreviations: BMS = Black Mexican Sweet maize suspension cultures; MS = Murashige and Skoog salts; GUS = β-glucuronidase; 2,4-D = 2,4 dichlorophenoxyacetic acid.

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Introduction

Development of simple and routinely applicable methods for DNA delivery into intact plant cells would greatly facilitate investigations involving transient expression or genetic transformation. DNA delivery into cells would obviate protoplasts as the target for DNA delivery experiments. Of the procedures for DNA delivery into intact cells, microinjection is technically demanding and can deliver DNA to only a few cells per experiment (Crossway et al. 1986). Acceleration of DNA-coated microprojectiles into intact plant cells has been used for DNA delivery into soybean (McCabe et al. 1988), tobacco (Klein et al. 1988), cotton (Finer and McMullen 1990), maize (Klein et al. 1987 and 1989), and wheat (Wang et al. 1988). Although particle acceleration has allowed the circumvention of the major constraints of methods such as protoplast isolation and microinjection, preparation of DNA-coated particles is relatively laborious and the cost of bombardment apparati may be prohibitive. *Agrobacterium*, an important transformation vector for dicotyledonous species, is of limited use for monocot transformation. These limitations have encouraged development of simple, rapid and less expensive DNA-delivery methods applicable to all plants.

Recently, Cockburn and Meier (unpublished) developed a procedure for injection of insect embryos using silicon carbide fibers. This method involves vortexing in an Eppendorf tube a mixture of plasmid DNA encoding a selectable or screenable marker gene, silicon carbide fibers, and eggs of the species to be transformed. The silicon carbide fibers apparently act as microinjection needles and facilitate DNA delivery into the insect embryos. This procedure is rapid, simple and inexpensive. The objective of this study was to determine whether silicon carbide fibers could be used to deliver DNA into plant cells.

Materials and Methods

Cell Suspension Cultures: Black Mexican Sweet (BMS) maize (*Zea mays* L.) cell suspension cultures were grown in liquid MS2D medium (Green 1977) containing MS salts (Murashige and Skoog 1962), with 150 mg/l asparagine, 0.5 mg/l thiamine-HCl, 20 g/l sucrose, and 0.5 mg/l 2,4-D, adjusted to pH 5.8 prior to autoclaving. Cultures were subcultured by 20-fold dilution into fresh MS2D medium approximately every 7 d. Cells to be used as samples were collected 5 to 7 d following subculture.

Regenerable, embryogenic maize suspension cultures were established by culturing friable, embryogenic callus in liquid N6 medium (Chu et al. 1975) containing 1 mg/l 2,4-D, 25 mM L-proline, and 100 mg/l casamino acids. Regenerable suspension cultures were subcultured by inoculating 7.5 ml of cell culture into 35 ml of fresh N6 medium approximately every 7 d. Friable, embryogenic callus used for suspension culture initiation was established from immature F_1 and F_2 embryos of the cross A188 x B73 (Armstrong and Green 1985).

Tobacco suspension culture TXD was kindly provided by Monsanto Co. Cultures were grown in liquid MSB5 medium containing MS salts, B5 vitamins (Gamborg et al., 1968), 30 g/l sucrose, 5 µg/l kinetin, 200 mg/l inositol, 130 mg/l L-asparagine and 4 mg/l parachlorophenoxyacetic acid,

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adjusted to pH 5.7. Cultures were subcultured by inoculating 10 ml of cell culture into 50 ml of fresh medium every 3 to 4 days.

Plasmid DNA: Plasmid DNA (pNGI) was provided by Dr. Michael Fromm, USDA Plant Gene Expression Center, Albany, CA. pNGI contains the β-glucuronidase (GUS) (Jefferson et al. 1987) gene under the control of the maize alcohol dehydrogenase (Adh1) promoter and intron I of maize Adh1 (Klein et al. 1989). Plasmid pBI221 was obtained from Clonetech Laboratories Inc. (Palo Alto, CA 94303). pBI221 contains the CaMV 35S promoter-GUS-NOS poly A signal cloned into pUC19 (Jefferson, 1987).

DNA Delivery: All preparations and treatments were performed aseptically. An aliquot of 1μg/μl plasmid DNA was combined with an aliquot of a 5% (w/v) suspension of silicon carbide fibers (Silar SC-9, ARCO Metals) in a 1.5 ml Eppendorf centrifuge tube. The fibers averaged 0.6 μm in diameter and 10-80 μm in length. The suspension was mixed by vortexing for 5 s. Suspension culture cells were collected using vacuum filtration, rinsed with fresh medium, and approximately 250 μl packed volume of cells was added to each Eppendorf tube containing the plasmid DNA and suspended silicon carbide fibers. Fresh culture medium (100 μl) was added to the mixture. The Eppendorf tube was capped, vortexed in an inverted position for 5-10 seconds, then returned upright and vortexed at top speed for 60 seconds using a desktop vortex unit (Vortex Genie 2, Scientific Industries, Inc.).

Experimental controls were replicated samples consisting of: 1. cells + medium; 2. cells + medium + DNA; and 3. cells + medium + silicon carbide fibers.

Maize and tobacco suspension culture cells were treated with pNGI and pBI221, respectively.

GUS Assays: Following vortexing, the contents of each tube were transferred to 60 x 20 mm disposable petri plates, 500 μl of fresh MS2D (BMS), N6 (regenerable maize) or MSB5 (tobacco) medium was added, and each plate was sealed with parafilm. Plates were incubated in the dark at 28°C for 2 d. Following the incubation period, histochemical assays of each sample were performed by adding 750 μl of histochemical GUS assay solution (Jefferson 1987) modified according to McCabe et al. (1988). The plates were incubated for 48 h at 28°C before observation for transient expression of the GUS enzyme. Transient expression events (blue cells or cell colonies) were identified visually under a 10X binocular microscope and counted.

Scanning Electron Microscopy: Nonvortexed BMS cell samples, and samples vortexed for 60 s with plasmid DNA + silicon carbide fibers + medium, or with DNA + medium only were each fixed in 1 ml of a solution of 2.5% (w/v) glutaraldehyde in 0.05 M phosphate buffer (pH = 7.0) overnight at 4°C. The following day, samples were washed with phosphate buffer, placed in 2% phosphate buffered solution for 30 min, and then washed three times with distilled water. After the final wash with distilled water, the samples were dehydrated by progressively placing the cells in solutions of 25%, 50%, 75%, 95%, and 100% (v/v) acetone/water for 10 min in each solution. Critical point drying of the samples was performed using liquid CO_2 in a vacuum evaporation unit. After the samples were dried, each was affixed to a specimen mount with double sided adhesive tape and coated with gold-paladium. The specimen mounts were placed on the stage of a Philips scanning electron microscope model 500, and the samples visualized using a 6 kV electron beam.

Disposal of Silicon Fibers: Silicon carbide fibers may be carcinogenic because they have properties similar to asbestos. Fibers were always maintained as a suspension in water to avoid respiratory exposure. Solutions and materials contaminated with the fibers were sealed in double plastic bags and stored in a vented hood until disposal.

Results and Discussion

Scanning Electron Microscopy: Black Mexican Sweet maize suspension culture cells which had not been vortexed (data not shown) and BMS cells vortexed in the presence of medium + DNA or medium + plasmid DNA + silicon carbide fibers were similar in appearance (Figures 1A and B).

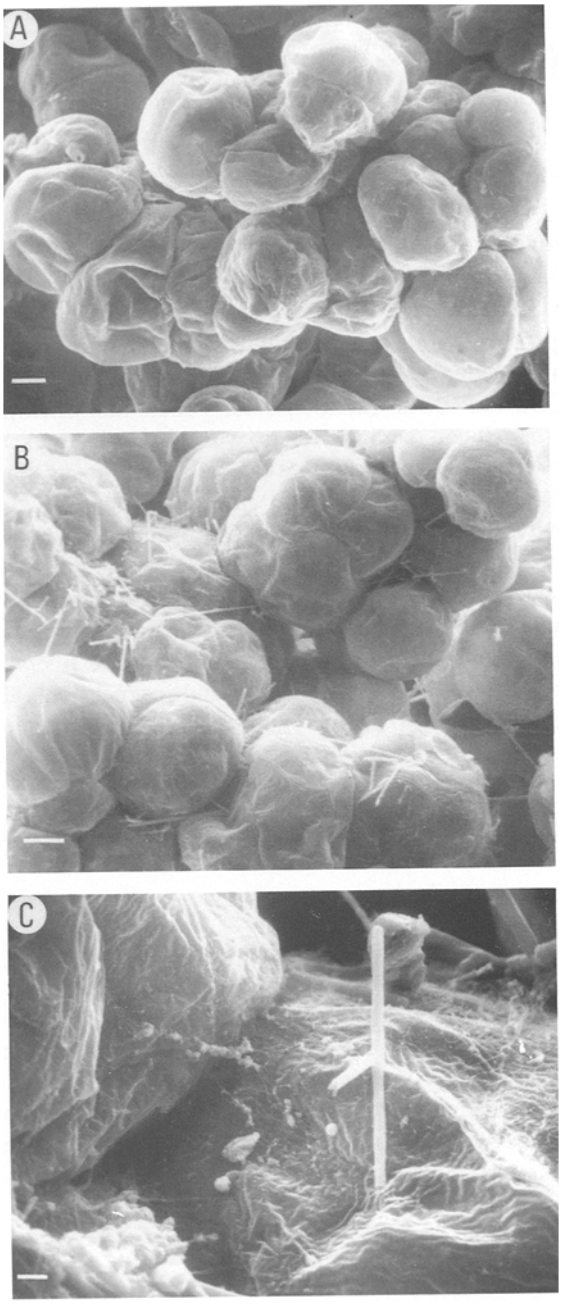

Fig. 1. Scanning electron micrographs of Black Mexican Sweet (BMS) maize suspension culture cells. Bar = 10 μm.

A. BMS suspension culture cells vortexed with MS2D medium + plasmid DNA (pNGI).

B. BMS suspension culture cells vortexed with MS2D medium + plasmid DNA (pNGI) + silicon carbide fibers.

C. Silicon carbide fiber protruding from BMS cell.

These results indicated that treatment with silicon carbide fibers did not increase disruption of cell walls and membranes. Figure 1C shows a silicon carbide fiber that may have pierced the wall of a maize cell. It was difficult to be certain that such fibers actually penetrated the cell because cells could not be viewed internally. Expression of GUS activity only when cells were treated with silicon carbide fibers + pNGI (see below) indicated that some fibers must pass through the cell wall to allow DNA delivery. It seems likely that DNA adhering to the fibers or to the cell wall is carried into the cell as the fiber is forced into a cell during collisions between cell colonies in the vortexed milieu.

GUS Assays: GUS activity was reproducibly observed in all samples of BMS, regenerable maize, and tobacco suspension culture cells that had been vortexed in the presence of culture medium, plasmid DNA and silicon carbide fibers. BMS cells treated with pNGI expressing the GUS gene are shown in Figure 2. Average frequencies of GUS expression units per sample following various treatments are listed in Table 1. The mean frequency of GUS expression units in samples of treated BMS cells was 139.5. Smith et al. (1984) estimated that a BMS cell density was 10^7 cells per g fresh weight. Therefore, the 250 µl packed volume samples of BMS cells used in these experiments were predicted to contain approximately 2.5 x 10^6 cells resulting in an estimated average frequency of transient expression of 10^{-4} per treatment.

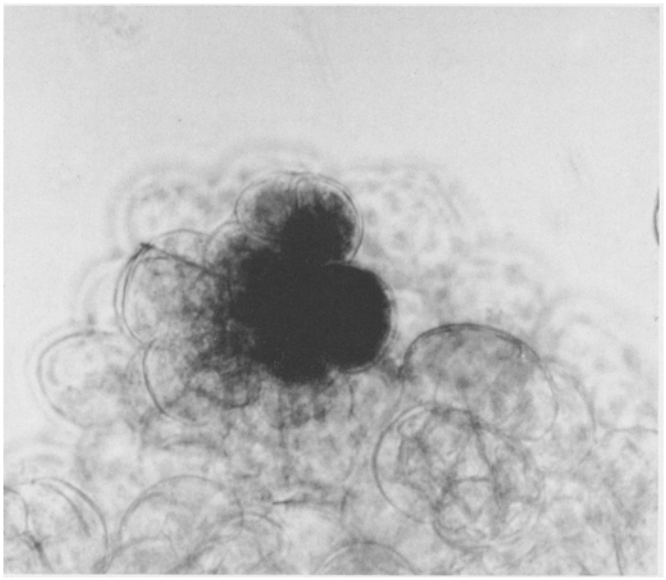

Fig. 2. Expression of GUS gene in cells of Black Mexican Sweet maize suspension culture following vortexing with pNGI + silicon carbide fibers.

Table 1. Treatment parameters and mean frequencies of GUS expression units in cells of Black Mexican Sweet (BMS), regenerable maize and tobacco suspension cultures.

Cell Culture	Volume of DNA[1] (µl)	Volume of fibers[2] (µl)	Mean # GUS expression units[3]	Range GUS expression units
MAIZE				
BMS	0	0	0	---
BMS	25	0	0	---
BMS	0	40	0	---
BMS	25	40	139.5	100-201
F_1-A188xB73	25	40	12.0	6-23
F_2-A188xB73	25	40	9.4	1-23
TOBACCO				
TXD	25	0	0	---
TXD	0	40	0	---
TXD	25	40	373.0	115-641

[1]concentration of DNA solution = 1 mg/ml.
[2]5% (w/v) suspension of silicon carbide fibers.
[3]1 GUS Expression Unit = one blue cell or one group of adjacent blue cells.

Treatment of cells from two different regenerable maize suspension cultures with fibers + pNGI resulted in mean frequencies of 12.0 and 9.4 GUS expression units per sample (Table 1). The difference in frequencies of GUS expression units observed in the different maize cell suspension cultures may be due to differences in cell wall thickness and composition, or other unknown factors. Tobacco suspension culture cells treated with fibers + pBI221 and exhibited GUS expression at a mean frequency of 373 GUS expression units per sample. We are investigating ways to increase DNA delivery frequency in all of the suspension cultures, and testing the method on other species as well.

None of the BMS or tobacco control samples listed in Table 1 expressed GUS activity. Expression of the GUS enzyme was also absent in additional controls consisting of nonvortexed BMS cells + medium, and BMS cells vortexed with medium + fibers + plasmid DNA which did not contain the GUS gene. We have treated hundreds of cell samples during investigations of the effects of different variables such as vortex time, DNA concentrations, and amount of silicon carbide fibers on frequency of GUS expression units per sample. Transient expression of GUS was observed in all samples vortexed with plasmid DNA coding for the GUS gene and silicon carbide fibers. Transient expression of GUS was not observed in any of the controls for these experiments and we have never observed non-specific GUS expression in BMS cells in our other work with particle acceleration (data not shown). The silicon carbide fiber-mediated DNA delivery procedure that is described in the materials and methods resulted in the most reproducible results and highest average of GUS expression units per sample of BMS and tobacco cells. These results indicated that the silicon carbide

fibers-vortex procedure can be used to rapidly and inexpensively introduce foreign DNA into intact plant cells for investigations of transient gene expression. Research to further characterize the mechanism of silicon carbide fiber-mediated DNA delivery to identify fibrous materials that pose less potential risk to the user seems warranted based on our results.

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